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Combined Atomic Force Microscopy and Optical Microscopy Measurements as a Method To Investigate Particle Uptake by Cells

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***W**e propose a combination of atomic force microscopy (AFM) and optical microscopy for the investigation of particle uptake by cells. Positively and negatively charged polymer microcapsules were chosen as model particles, because their interaction with cells had already been investigated in detail. AFM measurements allowed the recording of adhesion forces on a single-molecule level. Due to the micrometer size of the capsules, the number of ingested capsules could be counted by optical microscopy. The combination of both methods allowed combined measurement of the adhesion forces and the uptake rate for the same model particle. As a demonstration of this system, the correlation between the adhesion of positively or negatively charged polymer microcapsules onto cell surfaces and the uptake of these microcapsules by cells has been investigated for several cell lines. As is to be expected, we find a correlation between both processes, which is in agreement with adsorption-dependent uptake of the polymer microcapsules by cells.*

Keywords:
- adsorption
- cells
- cellular uptake
- imaging
- microcapsules

1. Introduction

Cells can incorporate objects ranging from the molecular up to the micrometer scale by processes including pino-, endo-, and phagocytosis.[1–7] Uptake of particles is preceded by a contact of the particle with the cell membrane. This contact can be of a nonspecific nature but can also be highly specific when ligands on the particle surface bind to membrane-bound receptors.[8] Consequently, a correlation between the particle's adhesion to the cell membrane and the rate of uptake can be expected. Cell membranes are charged objects, typically with a net negative charge, although positively charged domains exist.[9,10] Therefore, adhesion and, related to it, the uptake of particles should depend on the particle's charge. For an experimental analysis of charge-dependent adsorption, model particles have been used, such as ferritin[10,11] or hemeundecapeptide.[10,12]

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Studies on several systems showed an increased uptake of positively charged particles compared to that of negatively charged particles.[6,13,14] This could be explained by enhanced adsorption due to electrostatic interaction between the positively charged particles and the predominantly negatively charged cell membrane. Studies with fluorescent colloidal semiconductor nanoparticles also suggest this interpretation.[15] While the uptake rate of particles is relatively easy to access experimentally, it is more complicated to quantify the strength of adsorption to the cell membrane.

The aim of this study is to get quantitative numbers for the adhesion forces of charged particles to cell surfaces and for the rate of particle ingestion by the cells and to compare both values with regard to correlation. For this purpose we have chosen polymer microcapsules[16] as a model system. These microcapsules are composed of several onion-like layers of oppositely charged polymers. By a change of the outermost layer, they can be made either positive or negative.[16] The use of these microcapsules as delivery systems of pharmaceutical agents into cells is under investigation.[17] Due to their micrometer size, the capsules are easily experimentally accessible. We have measured the adhesion rate of the microcapsules by using atomic force spectroscopy.[18–20] The rate of uptake was determined by counting the number of ingested microcapsules inside the cells with optical microscopy.[17] Experiments were performed for negatively and positively charged microcapsules and for two different cell lines.

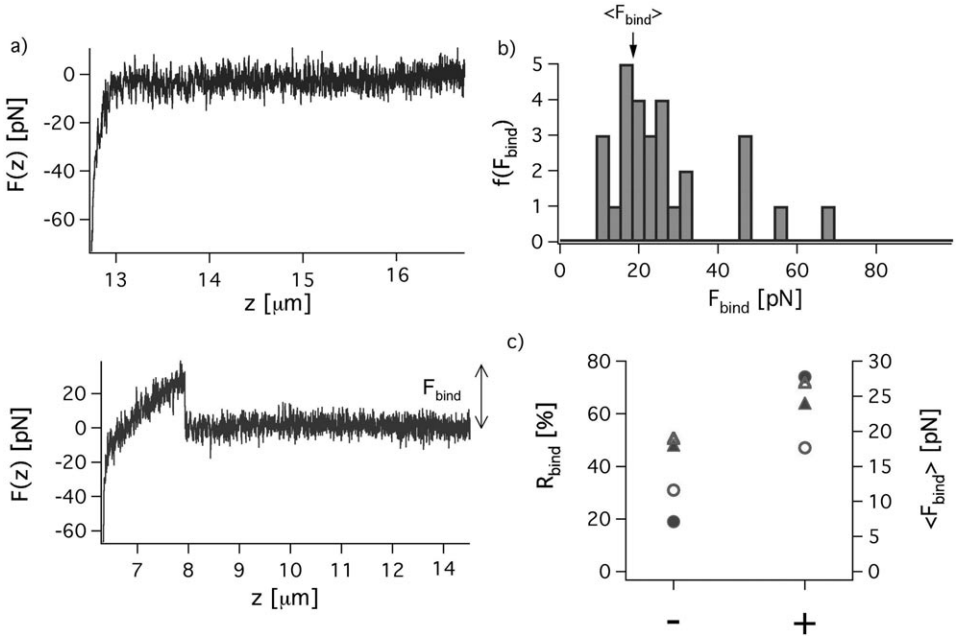

Figure 1. Results of the AFM measurements. a) Force–distance, $F(z)$, curves recorded for MCF-10A cells probed with a negatively charged microsphere. z refers to the distance between the surface of the cell and the surface of the microsphere plus an arbitrary constant. In the upper image, no bond between the cell and the microsphere has been formed, whereas in the lower image a bond has formed which ruptures upon pulling the microsphere away from the cell surface. The rupture force is depicted as F_{bind}. b) Histogram for the recorded rupture forces in the case of bond formation between the cell and the microsphere. $f(F_{bind})$ refers to the frequency with which rupture forces F_{bind} have been observed. $<F_{bind}>$ is the mean rupture force as determined from the histogram. c) R_{bind} (data points shown as circles) and $<F_{bind}>$ (data points shown as triangles) for different cells (MCF-10A/MDA-MB-435S) and microcapsules (positively/negatively charged). Data for MCF-10A cells are plotted in dark gray; data for MDA-MB-435S cells are plotted in light gray. The R_{bind} value is the percentage of recorded force–distance curves in which the formation of a bond between cell and microsphere has been observed.

2. Results and Discussion

2.1. AFM Measurements:

Two typical force–distance $F(z)$ curves are shown in Figure 1a. Whereas no bond between the microcapsule and the cell membrane had been formed in the case shown in the upper diagram, the rupture of a single bond between microsphere and cell can be observed in the case shown in the lower diagram. The rupture force is displayed as F_{bind} in the diagram. In Figure 1b, the histogram for the distribution of the measured rupture forces is displayed for the case of MCF-10A cells probed with negatively charged microspheres. From this diagram, the mean rupture force, $<F_{bind}>$, was determined. In this way, two data points were derived from the set of force–distance curves recorded for each cell line and each type of microcapsule: The percentage of $F(z)$ curves in which bond formation between the microsphere and the cell surface was found (R_{bind}), and the mean deadhesion force ($<F_{bind}>$) in the cases of bond formation. The resulting data are displayed in Table 1 and Figure 1c.

An obvious trend can be seen from these data. For both cell lines the adhesion rate and the adhesion force are higher for the positively charged microspheres than for the negatively charged ones, with the effect being more pro-

Table 1. a) Adhesion rate, R_{bind}, in % and b) mean deadhesion force, $<F_{bind}>$, in pN, recorded for two different cell lines (MCF-10A, MDA-MB-435S) that have been probed with negatively and positively charged microspheres. See also Figure 1.

a)	negative	positive
MCF-10A	31	47
MDA-MB-435S	19	74
b)	**negative**	**positive**
MCF-10A	19	27
MDA-MB-435S	18	24

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nounced for MDA-MB-435S cells. This trend is in agreement with the simplified picture of electrostatically promoted adhesion, that is, positively charged microspheres stick more strongly to negatively charged cell membranes than negatively charged microspheres. We have to point out again that this simplified global picture does not, of course, reveal any information about the mechanism of bond formation. However, it fits quantitatively with the AFM data in which bond formation between cells and microspheres has been investigated on a molecular level.

2.2 Uptake of Microcapsules by Cells: Counting Statistics of Incorporated Microcapsules

As has been described in a previous study, some cells have already incorporated some microcapsules after one hour of incubation with microcapsules.[17] We have also demonstrated that incubation with microcapsules at moderate concentrations does not show any significant effect on cell viability, as determined with an adhesion assay.[30] Cells with incorporated microcapsules preserve their ability to divide into two daughter cells (Figure 2), whereby the ingested microcapsules are passed to both daughter cells. This is similar to the behavior of cells with incorporated nanoparticles,[2] although the size of the capsules used here is in the micrometer range.

The aim of this study is to relate the number of ingested microcapsules to their surface chemistry. Therefore, the ability to count the number of microcapsules that have been incorporated by each single cell is a prerequisite. In previous studies we have claimed that MCF-10A, MDA-MB-435S, and NRK cells can internalize capsules of 5 μm diameter,[17,30] although the height of the cells lies in the same range.[31] Due to the very limited height resolution of conventional light microscopy, we found it rather difficult to distinguish between microcapsules just adsorbed on to the cell surface and microcapsules that have already been internalized by the cells, even after manually changing the focus. Therefore we employed confocal microscopy to obtain three-dimensional images of the cells and the microcapsules. As can be seen in Figure 3, cells indeed incorporate micrometer-sized capsules. We imaged cells after incubation with microcapsules by conventional light microscopy (Figure 4 shows an example image) and counted the number of microcapsules that we considered to be internalized by these cells. The same cells were imaged afterwards in the same set-up with confocal microscopy and three-dimensional images were reconstructed. From these images the actual number of internalized microcapsules could be determined with high reliability, since internalized microcapsules could be clearly distinguished from the ones adherent to the outside of the cells. Frequently, the number of microcapsules counted by conventional light microscopy was significantly higher than that determined by confocal microscopy. This means, that the number of internalized microcapsules as determined by conventional light microscopy is overestimated, as some microcapsules that are adsorbed to the outside of the cell membrane are de facto counted as internalized.

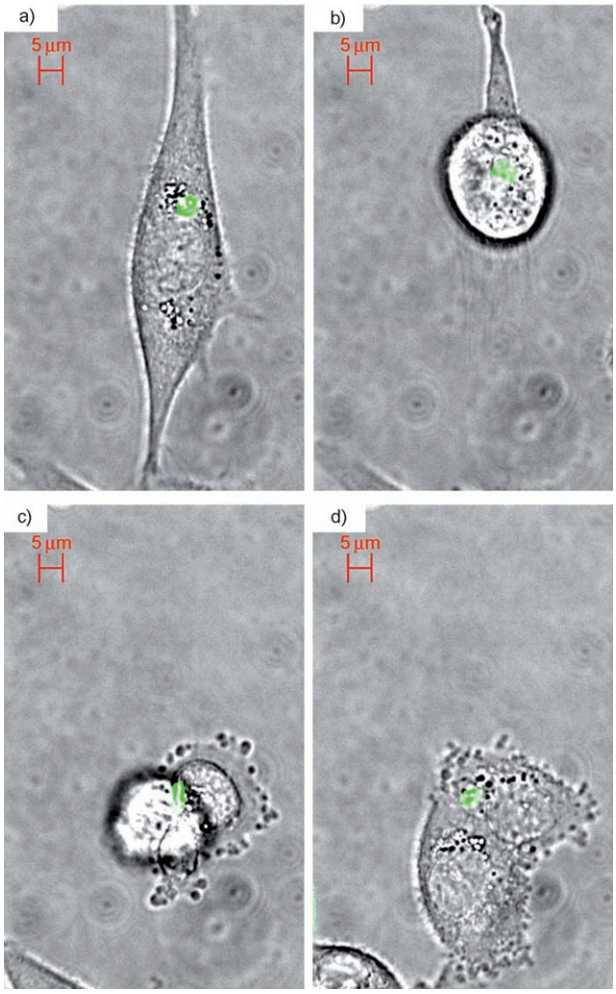

Figure 2. MDA-MB-435S cells have been incubated with positively charged polymer microcapsules of 5 μm diameter. During incubation a movie of the cells and microcapsules (with green-fluorescent CdTe particles in their walls) was recorded (1 photo every 8 min) with an optical microscope equipped with an incubation chamber. Overlays of phase-contrast and fluorescence images recorded after a) 880 min, b) 896 min, c) 1200 min, and d) 1432 min of incubation are shown. In this time interval, the cell divides and the ingested microcapsule is passed to one daughter cell.

There are three alternatives to obtain useful data about the number of actually incorporated microcapsules per cell. The first method is to use confocal microscopy. Unfortunately this method is much more laborious (staining of the cell membrane, recording of images at several focal planes, three-dimensional reconstruction) than conventional light microscopy. Secondly, addition of a quencher to the cell medium could quench the fluorescence of the microcapsules outside the cells. In this way only microcapsules inside the cells would preserve their fluorescence and all fluorescent microcapsules could be reliably counted as incorporated by conventional fluorescence microscopy.[6] Although this method has been used for some organic fluorophores, we were not able to find an appropriate biocompatible quencher for the CdTe nanoparticles used to fluorescence label the microcapsules in this study. Therefore, we have opted the

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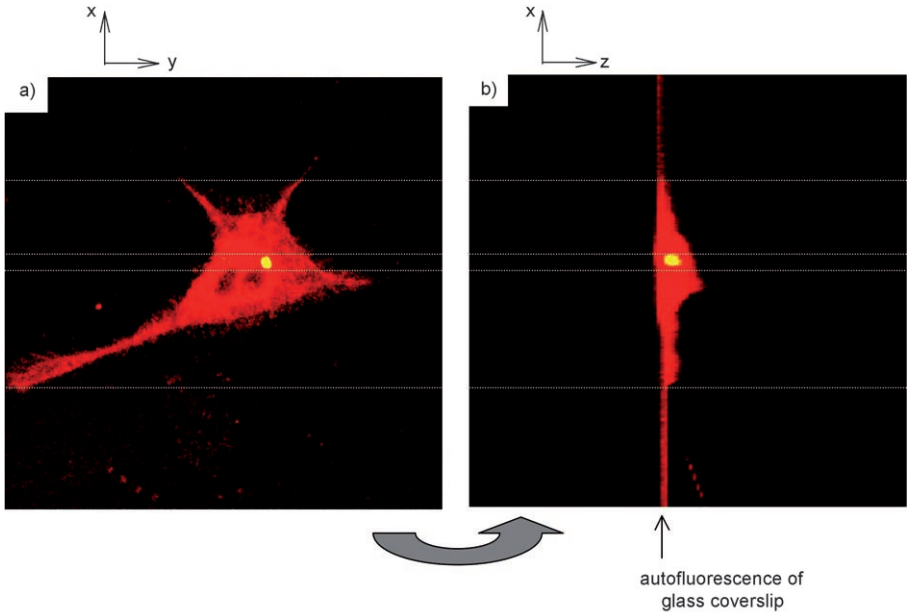

Figure 3. An MDA-MB-435S cell with one ingested positively charged polymer capsule of 5 μm diameter. The microcapsules have been fluorescence labeled with green-fluorescent Alexa and the membrane has been stained with red-fluorescent FM 4-64. The FM 4-64 also stained the microcapsules, which therefore appear in yellow as an overlay of the green and red. The overlay of the three-dimensional green- and red-fluorescence images that have been obtained with confocal microscopy is shown. a) The three-dimensional reconstruction of the cell as seen from above (that is, a view of the *xy* plane). b) The image from the same cell as seen from the side (that is, a view of the *xz* plane). The vertical red line through the whole image corresponds to the fluorescence of FM 4-64 adsorbed to the glass slide on which the cell has been cultured. Image (b) reveals that the microcapsule is indeed inside the cell.

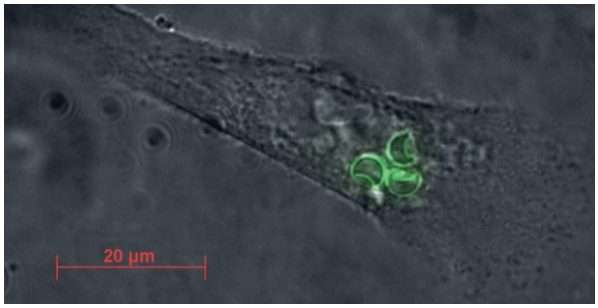

Figure 4. An MCF-10A cell with incorporated positively charged polymer microcapsules. The microcapsules were infiltrated with green-fluorescent CdTe nanoparticles. The overlay of the phase-contrast and fluorescence images is shown.

third alternative. By knowingly taking into account an overestimated number of internalized microcapsules, we decided to compensate for the uncertainty in the counting procedure by evaluating a huge number of cells.

By application of the same criteria to judge whether a microcapsule is counted as incorporated or not for all different types of microcapsules and cells, a relative comparison between the uptake rates should yield reliable numbers, although the absolute number of counted microcapsules will certainly be overestimated. Therefore, the data presented here have to be considered valid only for a relative comparison between the different systems. For each system, the number of ingested microcapsules was counted for at least 1000 different individual cells. To avoid systematic errors connected to the experimenter or the particular batch of cultured cells/microcapsules, we used for each system at least two different batches of microcapsules and at least four different batches of cultured cells. In addition, microcapsules were counted by three different persons. Similarly to the selection criteria reported in previous studies,[17,30] only microcapsules distributed around the nucleus and in its focal plane (as determined by manually changing the focus) were considered as internalized. A representative photo is shown in Figure 4. We note that sometimes the cells were deformed at the region around the ingested microcapsules.[30] Also, some of the internalized microcapsules were deformed (Figure 4).[17] At this point, the detailed mechanism of uptake remains unclear. However, we speculate that a significant part of the microcapsules is squeezed upon incorporation, thereby resulting in a reduction of size.

Figure 5a shows the histogram $f(N_{in})$ for the number of positively charged microcapsules that have been ingested by single MCF-10A cells. For better visualization, the cumulative probability, $p(N_{in})$, of this distribution is plotted in the same diagram. From the diagram, it can be seen that after one hour of incubation with microcapsules, more than 50% of cells did not have any incorporated microcapsules. To compare the results for different systems, cumulative probability plots of MCF-10A and MDA-MB-435S cells with negatively and positively charged microcapsules are shown in Figure 5b. Both cell lines incorporated more positively charged microcapsules than negatively charged ones during the same interval of incubation. The difference in ingestion rate between the oppositely charged microcapsules is bigger for the MDA-MB-435S cells than for the MCF-10A cells. As can be seen in Figure 5c and Table 2, after one hour of incubation only $p(N_{in}=0)=44\%$ of the MDA-MB-435S cells had not ingested any positively charged microcapsules, whereas $p(N_{in}=0)=82\%$ of the same cells had not ingested any negatively charged microcapsules.

The findings demonstrate that there is a remarkable nonspecific uptake of microcapsules by cell lines. Therefore, in order to investigate receptor-mediated uptake, first the nonspecific uptake has to be reduced. This could be done,

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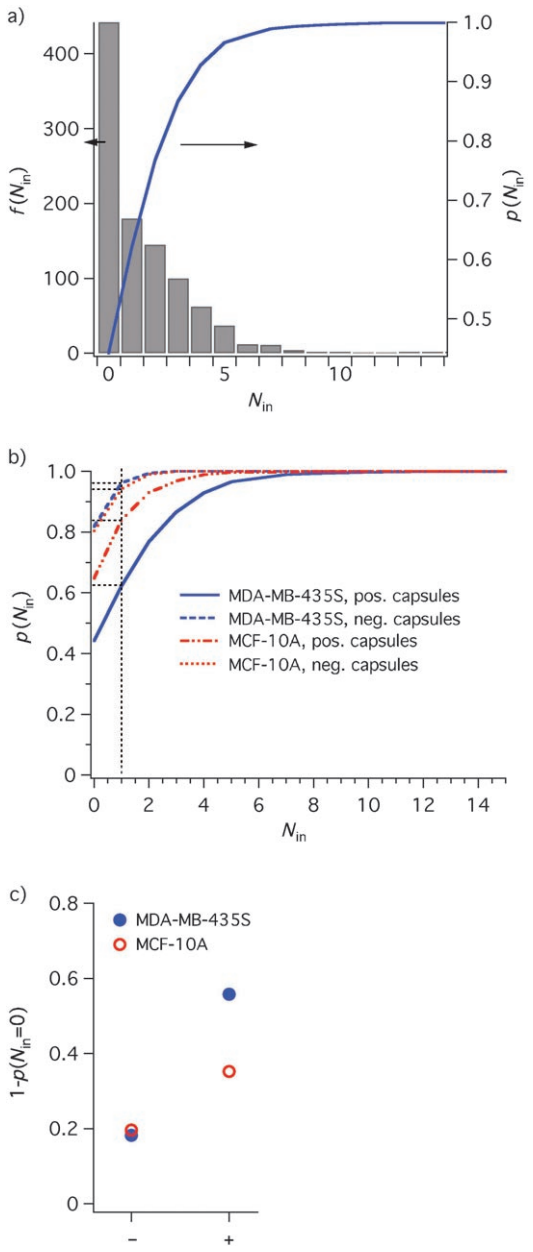

Figure 5. The number of ingested microcapsules per cell after one hour of incubation. a) Left scale: histogram for the number of ingested positively charged microcapsules per MDA-MB-435S cell. $f(N_{in})$ is the number of the cells that have incorporated N_{in} microcapsules. Right scale: for better visualization, the data of the histogram have been converted to a cumulative probability plot. $p(N_{in})$ corresponds to the normalized integral of the $f(N_{in})$ histogram.[32] In fact, $p(N_{in})$ is the probability that cells have ingested $\leq N_{in}$ microcapsules. b) Cumulative probability plots for MDA-MB-435S and MCF-10A cells that have ingested positively and negatively charged polymer capsules of 5 μm diameter. The trace for the MDA-MB-435S cells and positively charged microcapsules corresponds to the histogram shown in (a). c) $1-p(N_{in}=0)$ taken from the plot shown in (b). Data for MDA-MB-435S cells are shown in blue; data for MCF-10A cells are shown in red. The charge of the microcapsules is indicated by "+" and "−". $1-p(N_{in})$ corresponds to the probability with which cells have ingested $>N_{in}$ microcapsules. This means that $1-p(N_{in}=0)$ is the probability that cells have ingested at least one microcapsule per cell, whereas $p(N_{in}=0)$ is the probability that cells have ingested no microcapsules. $1-p(N_{in}=1)$ is the probability that cells have ingested at least one microcapsule per cell, whereas $p(N_{in}=1)$ is the probability that cells have ingested less than 2 microcapsules. The data are also shown in Table 2.

Table 2. Percentage of cells that have a) ingested at least one microcapsule per cell ($1-p(N_{in}=0)$ in %) and that have b) ingested at least two microcapsules per cell ($1-p(N_{in}=1)$ in %) after one hour of incubation, recorded for two different cell lines (MCF-10A, MDA-MB-435S) that have been probed with negatively and positively charged microspheres. See also Figure 5.

a)	negative	positive
MCF-10A	20	35
MDA-MB-435S	18	56
b)	**negative**	**positive**
MCF-10A	5.8	16
MDA-MB-435S	3.8	38

for example, by coating the microcapsules with poly(ethylene glycol) (PEG).[5] Ligands specific to receptors on the cell membrane could then be added to the shell of PEG.

2.3. Comparison of AFM and Uptake Experiments

The findings of the uptake study correspond well to the results obtained by AFM measurements (Figure 1c). We can conclude that, for short periods of incubation, there is a correlation between the probability of adsorption of a microcapsule to the cell membrane and the probability of internalization of the microcapsule. However, we have to point out that while the maximum contact time between the microcapsule and the cell surface is very short during AFM measurements (a few milliseconds), it can be up to one hour during the counting studies performed with optical microscopy. In total, for all investigated cell lines, both the adhesion rate and the adhesion force, as well as the number of ingested microcapsules, were higher for positively charged microcapsules than for negatively charged ones. This trend fits with the simplified picture of electrostatically promoted adhesion: positively charged capsules stick more strongly to overall negatively charged cell membranes than negatively charged ones and thus are ingested at a higher rate.

It has already been demonstrated in a previous report[17] that the number of internalized microcapsules per cell increases with time until saturation is reached. In this study, we have incubated MCF-10A, MDA-MB-435S, and NRK cells with positively charged microcapsules for 48 h. The result is shown in Figure 6. After 48 h, there are basically no cells that have not ingested at least one microcapsule ($p(N_{in}=0)\leq 5\,\%$). Interestingly, the differences in the number of ingested microcapsules between the three types of cells become negligible (compared to the experimental error) after 48 h of incubation (see Figure 6), whereas a clear difference could be seen after only 1 h of incubation between MCF-10A and MDA-MB-435S cells (see Figure 5b). This pattern could be explained by different kinetics of the respective uptake pathways. Alternatively, it might be ascribed to shielding of the microcapsule surface by non-specifically adsorbed constituents from the cell culture medium, so that previous differences in the surface chemistry of the microcapsules are smeared out.

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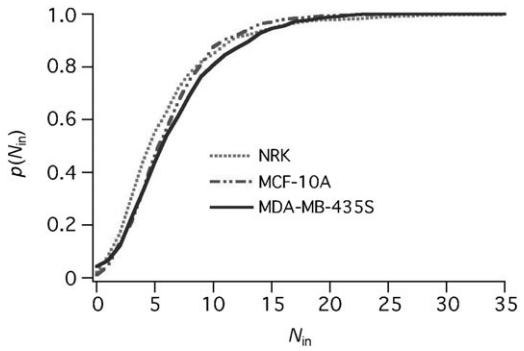

Figure 6. Cumulative probability plot of the number of ingested capsules for several cell lines that have been incubated for 48 h with positively charged polymer microcapsules. $p(N_{in})$ is the probability that cells have ingested $\leq N_{in}$ microcapsules per cell.

3. Conclusion

We conclude that polymer microcapsules are a suitable system to study cellular uptake mechanisms. This has been demonstrated in this study with a model system with already known interactions. The interesting feature of our system lies in the fact that, while adhesion to cells can be measured on a molecular level in terms of single bonds with AFM, the microcapsules are big enough to be visualized and counted by conventional light microscopy. With this system it should be possible to investigate surfaces that are more relevant for biological processes and thus obtain new knowledge about the processes with which cells ingest particles.

4. Materials and Methods

Microcapsule preparation: Polymer microcapsules of approximately 5 μm diameter have been assembled by coating a template melamine formaldehyde core of 4.88 μm diameter (10 % (v/v) aqueous suspension; Microparticles GmbH, Germany) successively with five alternating bilayers of negatively charged poly(styrenesulfonate) (PSS, $M_w = 70\,000\ g\,mol^{-1}$; Aldrich) and positively charged poly(allylamine hydrochloride) (PAH, $M_w = 70\,000\ g\,mol^{-1}$; Aldrich).[16] Polyelectrolyte concentrations were $2\ mg\,mL^{-1}$ in 0.5 M NaCl. The cores were removed by dissolution in 0.1 M HCl. For visualization purpose, green-fluorescent CdTe nanoparticles (3 nm diameter, stabilized by thioglycolic acid[21]) have been incorporated into the walls of the microcapsules by a previously reported method.[22] To define the net charge of the microcapsules, two (PSS/PAH) or three (PSS/PAH/PSS) additional polyelectrolyte layers were deposited after the CdTe nanoparticle infiltration, thereby rendering the surface either positive or negative, respectively. The microcapsules were washed three times with water before use.

Cell culture: We have used invasive MDA-MB-435S and noninvasive MCF-10A breast cancer cells and normal rat kidney (NRK) fibroblasts (ATTC). Cells were grown on uncoated glass slides (LabTek II, Nunc) and were cultured according to the specifications of the provider.

Adhesion measurements with atomic force microscopy (AFM): Since the attachment of polymer microcapsules to the cantilever of the atomic force microscope was not straightforward in a reproducible way, we used an alternative system to emulate the microcapsule surface. Negatively charged polystyrene latex spheres of 10 μm diameter were glued with a tiny spot of epoxy glue to the tip of the microscope cantilevers and were then successively dipped into solutions of positively charged PAH and negatively charged PSS. According to the protocol for the microcapsule preparation this process was repeated five times to create a polyelectrolyte multilayer shell. The outermost layer determines the charge of the shell. During AFM experiments, the cantilever modified with the polymer-coated latex microsphere was lowered towards the surface of a single cell until a contact force of about 50 pN was reached. After making contact, the cantilever was immediately retracted until it was completely separated from the cell. This resulted in an average cell-to-microsphere contact of only a few milliseconds. During this short contact time adhesive bonds between the microsphere and the cell surface were formed with a probability of $R_{bind} = 20–80\,\%$, which can easily be observed in the force–distance curves recorded by AFM.[23] Under conditions of $R_{bind} < 40\,\%$, more than 90 % of the formed bonds could be ascribed to a single bond between the microsphere and the cell and only 10 % to multiple bonds.[19] We define the adhesion rate R_{bind} as the percentage of the force–distance scans in which the formation of a bond between the microsphere and the cell could be observed. We define the deadhesion force $< F_{bind} >$ as the mean value of the strength of this bond.[24,25] Each prepared microsphere was approached to and retracted from the surface of a MDA-MB-435S cell at least 100 times while force–distance curves were recorded. Afterwards, an MCF-10A cell was probed with the same microsphere another 100 times. With the next microsphere the order was changed so first the MCF-10A cells and then the MDA-MB-435S cells were probed. In total, at least 500 force–distance traces were recorded for each cell line (MDA-MB-435S and MCF-10A) and for each type of microsphere (negatively and positively charged). By pressing the microspheres in a controlled way with minimum force of around 50 pN to the surface of the cells, we ruled out the deformation of cells larger than 200 nm, although microspheres are more rigid than microcapsules.[26–29]

Confocal microscopy: To test whether microcapsules had been internalized by cells or were just adherent to their outer surface, images of cells that had been exposed to microcapsules were recorded with confocal microscopy (Zeiss Axiovert 200/LSM 5). Microcapsules labeled in their walls with green-fluorescent Alexa dye were used. After incubation of the cells with microcapsules for some hours, the free microcapsules were removed from the solution by carefully rinsing the cells. A red-fluorescent dye with high affinity to the cell membrane (FM 4-64; Molecular Probes) was then added according to the manual of the supplier. In this way, cells and microcapsules could be visualized in parallel by their red and green fluorescence, respectively, by using confocal microscopy. Images were recorded for several image planes and a three-dimensional image was obtained by convoluting the *z*-stack scans.

Counting of the number of internalized microcapsules with optical microscopy: Cells were seeded on uncoated glass cover slips and, after 12 h incubation time, microcapsules were added (32 microcapsules per seeded cell). After 1 or 48 h of incubation of the cells with microcapsules, images were recorded with phase-

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contrast and fluorescence microscopy (Zeiss Axiovert 200M). The number of internalized microcapsules per cell was counted by changing the focus .[17] As described in more detail in the Results section, this counting procedure has to be interpreted with care. We define the number of ingested microcapsules per cell, as defined by our criteria, as N_{in}. For each cell line and each type of microcapsule, at least 1000 different cells were analyzed by determining the number of internalized microcapsules. The distributions of N_{in} are visualized as histograms, $f(N_{in})$, and cumulative probability diagrams, $p(N_{in})$.

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